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ONTOGENY OF CORTICOTROPIN RELEASING HORMONE GENE EXPRESSION IN RAT HYPOTHALAMUS – COMPARISON WITH SOMATOSTATIN

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Abstract—Using *in situ* hybridization histochemistry, corticotropin-releasing hormone gene expression is first detectable in the parvocellular portion of the rat paraventricular nucleus on the 17th fetal day. The prevalence of messenger RNA for corticotropin releasing hormone decreases perinatally, specifically between the 19th and 21st fetal days. By the 4th postnatal day, CRH gene expression is similar to that of the adult rat. Somatostatin messenger-RNA is detectable on the 14th fetal day in the periventricular nucleus. No perinatal hiatus in somatostatin gene expression is evident.

Key words: mRNA, CRH, corticotropin releasing factor, somatostatin development.

Corticotropin releasing hormone (CRH), synthesized in the hypothalamic paraventricular nucleus (PVN), mediates the release of adrenocorticotropic hormone (ACTH) and corticosteroids in response to stressful stimuli, as part of the brain-pituitary-adrenal axis.[12] During the perinatal period in the rat, i.e. the stress non-responsive period, little perturbation of hormonal levels occurs in response to noxious events.[9] The relationship between the regulation of CRH gene expression during late fetal and perinatal periods, and the onset of the stress-non-responsive-period has not been elucidated. This study explores the ontogeny of CRH messenger ribonucleic acid (CRH-mRNA) in the rat diencephalon, in comparison to that of an unrelated neuropeptide, somatostatin.

EXPERIMENTAL PROCEDURES

Tissue Preparation

Time-pregnant Sprague–Dawley derived rats were obtained from Zivic-Miller (Zelienople, PA) at least two days prior to sacrifice. Rats were kept on a 12 hour light/dark cycle (lights on 7 a.m.–7 p.m.), and given access to unlimited lab chow and water. Pregnancy was dated by the presence of a vaginal plug (day 0). Gestation in these rats lasts for 21 days. Fetal brains were obtained daily starting on the 14th fetal day, and on postnatal days 1, 4, 19 and 100. Brains were obtained between 8:30 and 9:30 a.m. Prenatally, pregnant rats were anesthetized with CO_2, fetuses were quickly dissected and heads were removed onto powdered dry ice. Postnatally, pups were decapitated, and brains removed onto dry ice. Brains were stored at −80°C.

Brains were cut into 20 micron coronal slices in a cryostat (IEC, MA) and mounted on gelatin-coated slides. Brain regions were identified by established landmarks.[2,10] Sequential sections were cut from the anterior commissure/septum through the caudal hypothalamus.

Hybridization histochemistry

Prior to *in situ* hybridization (ISH), slices were brought to room temperature, air-dried and fixed for 20 minutes in fresh 4% buffered PBS-paraformaldehyde. Sections were dehydrated through increasing ethanol concentrations, rehydrated, exposed for 8 minutes to 0.5% acetic anhydride −0.1 M triethanolamine (pH = 8), then dehydrated through 100% ethanol.

ISH was modified from Young *et al.*,[13] as previously described.[3] Briefly, prehybridization for one hour in hybridization buffer (0.2 ml/slice) was followed by a 20 hour ISH at 37°C. Hybridization buffer consisted of: 50% formamide, 4 × SSC (1 × SSC is 0.15 M NaCl in 0.015 M sodium citrate, pH = 7), 0.5 gm/ml sheared, single-stranded salmon sperm DNA, 25 mcg/ml yeast tRNA,

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Abbreviations: ACTH, adrenocorticotropic hormone; CRH, corticotropin releasing hormone; ISH, *in situ* hybridization; mRNA, messenger ribonucleic acid; PVN, paraventricular nucleus; SCA, subcortical area.

100 mM DTT, 5 × Denhardt's solution and 10% dextran sulfate. Reaction volume was 0.03 ml/section, under a coverslip in a humidity chamber. ISH was followed by serial washes at 37°C: 4 × 15 minutes in 2 × SSC, then 30 minutes each in 1 × and 0.4 × SSC. The last two washes were at room temperature. Hybridized slices were dehydrated and apposed to film (Hyperfilm B-max, Amersham, IL) for 24 hours and developed in 80% solution D-19 Kodak developer.[8]

A 60 nucleotide synthetic probe corresponding to the codons for the 20 COOH-amino acids of CRH[3,11] was generated using an Applied Biosystems (Foster City, CA) DNA Synthesizer. After purification, the probe was labelled on the 3′ end with dATP-S^{35} (NEN, Wilmington, DE) using terminal deoxynucleotidyl transferase[7] (Bethesda Research Labs.). Specific activity of labelled probes was 5–8 × 10^8 dpm/mcg. The complementary 'sense' strand and the 39 oligonucleotide probe for somatostatin-mRNA,[6] were similarly generated and labelled.

Quantitation and statistical analysis

Serial sections of the PVN at each age were examined, and those with the maximal area of PVN, based on toluidine blue staining, were used for analysis. Films were mounted on a light table with a precision illuminator (Northern Light B90; Imaging Research, St. Catherine, Ontario, Canada). Images were acquired by a Sierra Scientific high resolution camera (Sunnyvale, CA), and optical density (OD) was determined using the MCID software image analysis system (Imaging Research, St. Catherine, Ontario, Canada). Optical density was determined over the paraventricular nucleus, as well as over the parietal cortex for CRH-mRNA assessment; OD over the latter was defined as background. For slices subjected to ISH for somatostatin-mRNA, OD was determined over the anterior, basal periventricular nucleus and over the subcortical area underlying the lateral ventricles of the same slice (SCA). Since even sections subjected simultaneously to ISH, processed together and apposed to the same sheet of film had different background optical density, the ratios of OD of PVN and parietal cortex were used for comparison for CRH. For the same reason, the OD ratio of periventricular nucleus to SCA was utilized as a measure of the prevalence of somatostatin-mRNA. Five ratios, derived from at least 3 animals from two separate litters, were obtained for each age group, and means and standard deviations were determined for each age. Analysis of variance (Minitab, University Park, PA), was used to assess the significance of differences between age groups. Brainpaste standards[13] were apposed to each film, to ascertain that all samples were in the linear range of OD/cpm.

RESULTS

Using *in situ* hybridization, CRH gene expression is not detectable in hypothalami of 16 day fetal rats. Sequential slices, spanning the region from the medial preoptic/anterior commissure caudally to the mammillary bodies are free of specific hybridization to CRH probe (Fig. 1).

CRH-mRNA is present in 17 and 18 day fetal rat brain slices in a globoid, symmetric paraventricular region measuring 80 microns in diameter, bordering on the 3rd ventricle (Fig. 2). By the

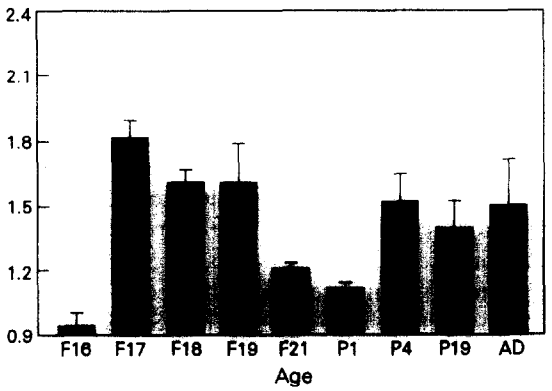

Fig. 1. Ontogeny of CRH gene expression in rat paraventricular nucleus. Results are expressed as means and standard deviations of 5 samples. See text for details of semiquantitative analysis. F16, etc., denote fetal days; P1, etc., denote postnatal days. AD = adult. Values of F21 and P1 are significantly ($P < 0.01$) different than those of F19 and P4.

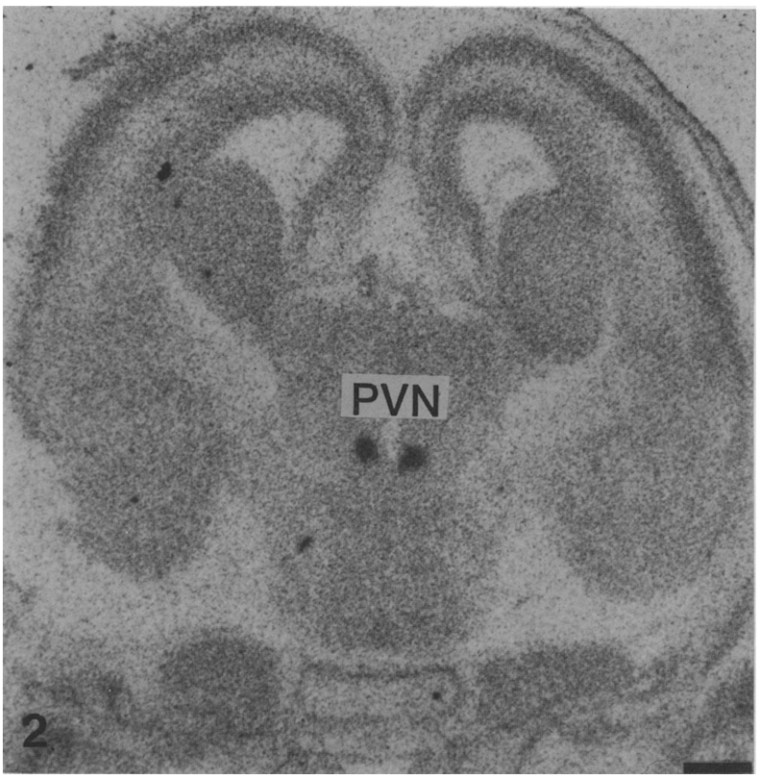

Fig. 2. Photomicrograph of paraventricular nucleus of a 17-day-old fetal rat subjected to ISH with S^{35}-labelled probe for CRH-mRNA. See Experimental Procedures for details of ISH. Bar × 1 mm. PVN = paraventricular nucleus.

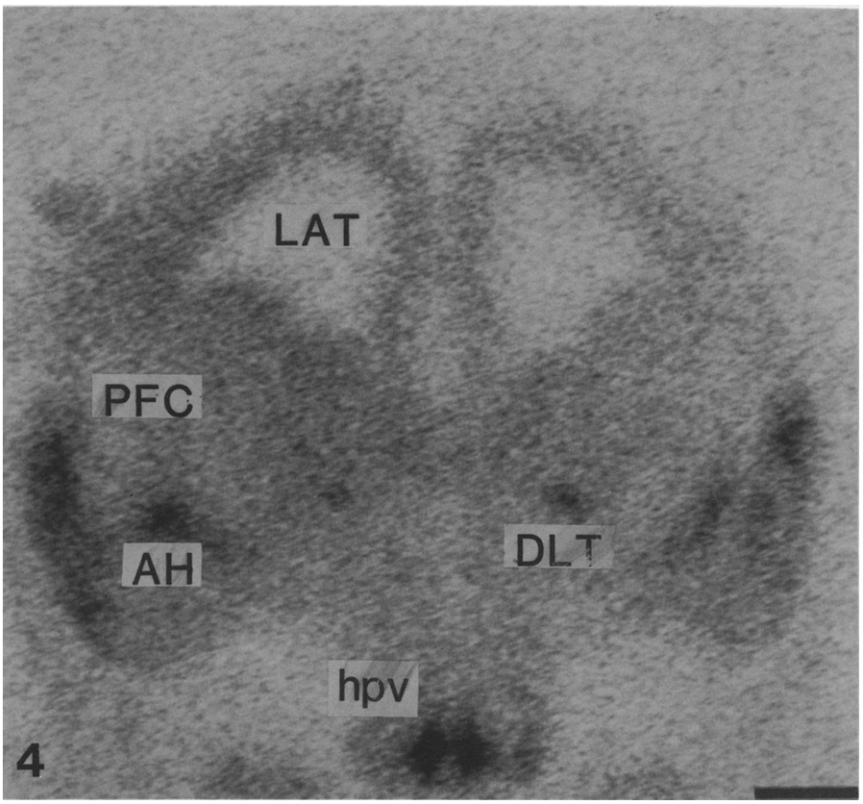

Fig. 4. Coronal section of the diencephalon of a 17-day-old fetal rat subjected to ISH with a labelled oligonucleotide complementary to Somatostatin-mRNA. Bar × 1 mm. AH × Amygdalo/hippocampal complex, hpv = periventricular nucleus, LAT = lateral ventricle, DLT = dorsolateral thalamus, PFC = parietofrontal cortex

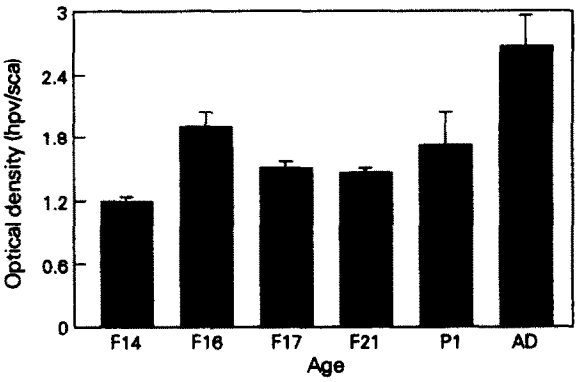

Fig. 3. Ontogeny of somatostatin-mRNA in the rat periventricular nucleus. Results are expressed as the means and standard deviations of 5 samples. F14, etc. denote fetal days; P1 is the first postnatal day. AD = adult. Value at F21 is not significantly different than those at F17 or P1

19th fetal day, the CRH-mRNA containing portion of the PVN spans 140 microns in the rostrocaudal dimension, and has begun to assume the more triangular shape of the mature parvocellular PVN. CRH gene expression is robust during the 17–19th fetal days, but decreases by the last, 21st fetal day. As seen in Fig. 1, CRH-mRNA is relatively abundant again by the 4th postnatal day, when the ratio of optical densities of PVN and cortex is similar to adult values.

Somatostatin gene expression is first evident in rat diencephalon on the 14th fetal day (Fig. 3). As evident from the figure, no perinatal reduction occurs in the prevalence of somatostatin-mRNA in the periventricular nucleus. Somatostatin-mRNA localization in the basal periventricular region (as well as in the hippocampo-amygdala complex and in circumscribed cortical areas) on the 17th fetal day, is shown in Fig. 4.

DISCUSSION

In this study, using adjacent sections of the same brains, we investigated the ontogeny of CRH and somatostatin gene expression in the rat diencephalon. Our findings of the appearance of ISH-detectable CRH-mRNA on the 17th fetal day is in agreement with those reported by Grino *et al.*,[5] who utilized ISH as well. Quite recently, Emanuel *et al.*,[4] employing northern blot analysis of total brain or hypothalamic RNA, reported the presence of CRH-mRNA as early as the 15th fetal day. These investigators did not find any perinatal decrease in the amount of CRH-mRNA, as opposed to Grino's results and those reported above. The basis for this discrepancy may well be the use of whole hypothalami: CRH-mRNA present in areas other than the PVN, (e.g., supraoptic or suprachiasmatic nuclei) may not fluctuate and may 'dilute' any changes seen solely in the PVN. A review of Fig. 2 in Emanuel's report[4] suggests a marked decrease in CRH-mRNA prevalence between the 15th fetal day and the subsequent samples obtained on the 20th and 21st fetal days.

The perinatal decrease in CRH-mRNA seems to be relatively specific for this peptide, since no such transient decrease was observed for somatostatin-mRNA. These findings are in agreement with published observations.[1,4] These investigators, using northern blot analysis, found the onset of somatostatin gene expression on the 15th and 14th fetal day, respectively. Both authors reported a steady increase in somatostatin-mRNA prevalence in rat hypothalamus from the late fetal age onwards.

In summary, we demonstrate the onset of CRH gene expression on the 17th fetal day in the rat PVN. CRH-mRNA prevalence decreases perinatally, then increases to adult values by the 4th postnatal day. Somatostatin-mRNA, present in rat diencephalon by the 14th fetal day, does not undergo a similar perturbation.

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